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Transgenic studies with a keratin promoter-driven growth hormone transgene: Prospects for gene therapy

(keratinocyte/skin/transgenic mice)

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ABSTRACT **Keratinocytes are potentially appealing vehicles for the delivery of secreted gene products because they can be transferred to human skin by the relatively simple procedure of grafting. Adult human keratinocytes can be efficiently propagated in culture with sufficient proliferative capacity to produce enough epidermis to cover the body surface of an average adult. However, the feasibility of delivering secreted proteins through skin grafting rests upon (*i*) the strength of the promoter in keratinocytes and (*ii*) the efficiency of protein transport through the basement membrane of the stratified epithelium and into the bloodstream. In this paper, we use transgenic technology to demonstrate that the activity of the human keratin 14 promoter remains high in adult skin and that keratinocyte-derived human growth hormone (hGH) can be produced, secreted, and transported to the bloodstream of mice with efficiency that is sufficient to exceed by an order of magnitude the circulating hGH concentration in growing children. Transgenic skin grafts from these adults continue to produce and secrete hGH stably, at ≈1/10 physiological levels in the bloodstream of nontransgenic recipient mice. These studies underscore the utility of the keratin 14 promoter for expressing foreign transgenes in keratinocytes and demonstrate that keratinocytes can be used as effective vehicles for transporting factors to the bloodstream and for eliciting metabolic changes. These findings have important implications for considering the keratinocyte as a possible vehicle for gene therapy.**

The epidermis and its appendages are self-renewing tissues, which have compartments of stem cells, each with enough proliferative capacity to cover the body surface of an individual (1). The pioneering studies of Green and coworkers (2, 3) established that human skin keratinocytes could be serially propagated in culture for several hundred generations. This led to the development of employing cultured skin keratinocytes in burn grafting operations, a technique that is now widely used in trauma units of major hospitals (4).

The accessibility of the skin and the proliferative capacity of cultured epidermal cells also makes keratinocytes ideal candidates for genetic manipulation and gene therapy (5–9). Gene transfer into cultured keratinocytes has been demonstrated by utilizing a variety of different foreign promoters to drive expression of various secreted products (5, 10–12). Because the phenotype and metabolic effects of increased growth hormone (GH) secretion are well characterized, GH has been widely used in these experimental studies. Cultured epidermal cells transfected with chimeric human GH (hGH) transgenes can produce and secrete more than 1% of the daily levels secreted by an equivalent mass of human pituitary gland (5). However, hGH is not detectable in the bloodstream of mice that receive these cultured keratinocyte grafts due to the inhibition of these foreign promoters over time (11, 12). The inactivation of these promoters *in vivo* has made it impossible to assess whether circulatory delivery of hGH from keratinocytes is a feasible avenue for gene therapy.

Keratin 14 (K14) and its partner K5 are the major proteins expressed by the mitotically active cells of the epidermis and its appendages (13), and the genes encoding these keratins are abundantly transcribed in cultured human keratinocytes (14). For these reasons, the K14 and K5 promoters are especially attractive candidates for use in keratinocyte-mediated gene therapy. In the present study, we explored the potential of using the human K14 promoter for gene therapy. Using a chimeric K14–hGH as our transgene, we examined the ability of transgenic skin keratinocytes to produce, process and secrete a 22-kDa hGH. We investigated the circulatory levels of hGH and the physiological responses of K14–hGH transgene expression in mice. Finally, we tested the ability of K14–hGH transgenic skin grafts to deliver systemic hGH to nontransgenic recipient mice. Our findings document that the K14 promoter can circumvent a major impediment encountered previously; namely, the inability of keratinocytes to sustain appreciable levels of gene expression by foreign transgene promoters integrated randomly in the genome.

MATERIALS AND METHODS

Preparation of Transgene Constructs and Generation of Transgenic Mice. An *Ava*I fragment containing 2100 bp of 5′ upstream sequence from the human K14 gene was used to drive expression of a β-galactosidase (β-gal) cDNA and the hGH gene (ref. 15; Fig. 1). Construction of the epitope-tagged K14 expression vector has been described elsewhere (16). Where indicated in Fig. 1, constructs also contained SV40 or AP2 intronic and SV40 3′-untranslated sequences and polyadenylylation signals to aid in the processing of transcripts (15, 16). Each reporter construct was microinjected into fertilized mouse eggs that were then used to generate transgenic mice. Transgenic mice were engineered in the outbred CD-1 strain

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Abbreviations: GH, growth factor; hGH, human GH; SV40, simian virus 40; β-gal, β-galactosidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RIA, radioimmunoassay; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; SCID, severe combined immunodeficient; RT-PCR, reverse transcriptase–PCR; K14P, neuropeptide substance P-tagged keratin 14; TNF-α, tumor necrosis factor α; MtI, metallothionine I.

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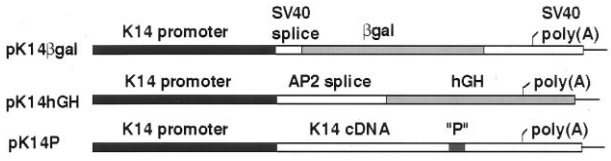

FIG. 1. Expression constructs used for the generation of transgenic mice. Stick diagrams depict the K14 promoter constructs engineered to drive expression of transgenes. Plasmid pK14βgal contains the 2.1-kb *Ava*I fragment containing the K14 promoter/enhancer, extending to the transcription initiation site. This fragment was followed by the intron present in the 5′-untranslated sequence of the simian virus 40 (SV40) large T antigen gene (SV40 splice), followed by a *Bam*HI site in which the β-gal gene was inserted, followed by the 3′-untranslated sequences and polyadenylylation site from the SV40 large T antigen cDNA (SV40 polyA). Plasmid pK14hGH is similar in design and yields an indistinguishable expression pattern from pKβgal, but contains 1.1 kb of sequences from the AP2 gene (AP2 splice) in its *Bam*HI site (these sequences did not interfere with hGH expression and are irrelevant for the purposes outlined in this paper). The entire 2.1-kb hGH gene followed, including introns, 3′-untranslated sequence, and polyadenylylation signal. An additional reporter construct was used, previously referred to as pH3cK14 promoter (16), and referred to here as pK14 promoter. Plasmid K14 promoter contained the K14 promoter, followed by the complete K14 coding sequence, modified at its 3′ end to add a small epitope tag sequence encoding the antigenic portion of neuropeptide substance P, followed by 3′-untranslated sequences and polyadenylylation signal of the K14 gene (16).

as described (16). For each construct, F_1 offspring from at least two independent founder animals were studied and shown to have comparable patterns of transgene expression.

Preparation of RNA and cRNA Probes for RNase Protection. Newborn, 2.5-week and 36-week-old adult transgenic and control mice were killed by cervical dislocation, and their tissues were frozen in liquid nitrogen and then pulverized. Tissue RNAs were prepared by extraction with TRIzol reagent (GIBCO) according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase (Promega) and stored at −70°C before use.

The DNA template for the synthesis of hGH antisense transcripts was generated by PCR with hGH cDNA (17). The template DNA begins 6 nt 5′ from the translation initiation codon in exon 1, encoding the hGH secretory peptide sequence, and ends at exon 3 encoding the hormone sequence. A portion of the T7 promoter sequence was included in the antisense-oriented primer and 13–15 additional random nucleotides were added at both ends of the amplified sequence. Radiolabeled antisense hGH and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (control) RNA probes were synthesized using a Maxiscript T7 kit (Ambion, Austin, TX).

RNase protection assays were carried out as described by Williams *et al.* (30) with reagents provided in the RNase Protection Kit (Boehringer Mannheim). Approximately 20–40 μg of tissue RNAs (constant for a matched set of control/transgenic) were hybridized with radiolabeled antisense RNAs (1×10^5 cpm). Following hybridization and RNase treatment, the protected RNA fragments were resolved by electrophoresis through 6% acrylamide gels containing 8 M urea.

Reverse Transcriptase–PCR (RT-PCR). Complementary DNA for PCRs was made using Moloney murine leukemia virus RT (Seikaguku America, Rockville, MD). One microgram of RNA was denatured at 95°C for 5 min in the presence of 2.5 μM random hexamers and 1 μM of a primer complementary to the RNA of interest. Reactions were then brought to 100 mM Tris·HCl (pH 8.0), 80 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM each dATP, dCTP, dTTP, dGTP, 40 units RNasin, 5 μM random hexamers, 200 units reverse transcriptase in 20 μl and incubated at 42°C for 60 min. Reactions were stopped by incubation for 5 min at 95°C and cooled immediately on ice. An aliquot (10 μl) of the reaction was then added to 40 μl of 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01%

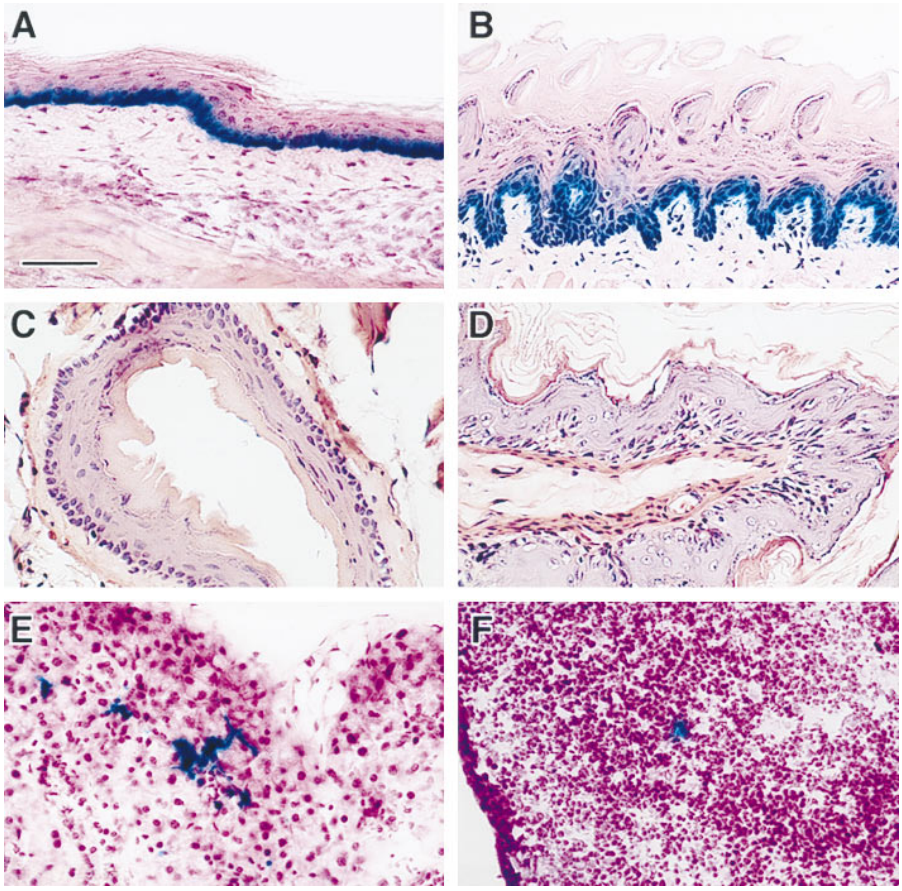

FIG. 2. β-Gal activity assays in tissue sections from pK14βgal transgenic mice. Transgenic mice positive for the pK14βgal transgene were generated as described. Tissues were collected and processed for β-gal activity assays and for hematoxylin/eosin counterstaining as described. Most of the data on transgene expression are compiled in Table 1. Shown are representative examples of sections from tail-skin (*A*), dorsal portion of tongue (*B*), esophagus (*C*), forestomach (*D*), salivary gland (*E*), and thymus (*F*). Note that for esophagus and forestomach, samples were also examined without counterstaining to verify that no β-gal activity was detected (not shown). Note also that only a few myoepithelial cells of the salivary gland and even fewer reticular cells of the thymic epithelium stained positive for the transgene. (Bar = 67 μm in *A–D* and *F*, and 42 μm in *E*.)

(wt/vol) gelatin, 1 unit *Taq* polymerase, and overlaid with light mineral oil. Reaction conditions: 94°C, 1 min denaturation; 62°C 1 min annealing; 72°C, 1 min chain elongation; 35 cycles. Samples were analyzed on 1.5% TAE (40 mM Tris·acetate/1 mM EDTA) agarose gels.

Keratinocyte Culture and hGH Radioimmunoassay (RIA). Keratinocytes were isolated by trypsinization of skins from newborn transgenic line 22 and littermate control mice. Keratinocytes were cultured in low calcium medium and supported with a mitomycin C-treated 3T3 fibroblast feeder layer (2, 19). When 100-mm dishes of keratinocytes were ≈80% confluent, 10 ml of fresh medium was added, and 24 hr later 200 μl was assayed for the presence of hGH. The level of hGH in the medium was measured by RIA with a Tandem-R hGH kit (Hybritech) according to the manufacturer's instructions. Keratinocytes from each dish were washed with versene to remove the 3T3 feeder cells and then trypsinized and counted using a hemocytometer.

For hGH detection in the bloodstream, sera were collected from 2.5- and 36-week-old transgenic and control littermates, and the concentration was determined by RIA.

Transgene Expression Assays. Backskins from newborn and 9 month old mice were placed in optimal cutting temperature (OCT) compound and frozen at −80°C. Frozen sections (8 μm) of skins were fixed with 3.7% formaldehyde for 10 min and then subjected to immunofluorescence immunohistochemistry using the following antibodies: for K14 promoter transgene detection, a rat monoclonal antibody against the neuropeptide substance P epitope tag (10); for hGH transgene detection, a mouse mAb against hGH (Sigma); for control basal layer staining, a guinea pig polyclonal antibody specific for keratin 5 (20) (data not shown). The fluorescein isothiocyanate-conjugated goat anti-mouse/rat or Texas Red-conjugated goat anti-guinea pig secondary antibodies were used with a Zeiss Axiophot immunofluorescence microscope to visualize the bound antibodies. For detection of β-gal activity, 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) conversion assays were used (15). Sections of tissue (≈10 μm) were fixed for 2 min in 0.5% glutaraldehyde, and β-gal activity was visualized by its ability to cleave X-Gal, thus producing a blue product (ref. 21 and references therein.) Sections were postfixed in 0.5% glutaraldehyde for 30 min and then counterstained with hematoxylin/eosin. To verify absence of β-gal activity, samples were also reacted, without counterstaining.

For *in situ* hybridizations, backskins of 3-month-old mice were fixed with 4% paraformaldehyde, mounted in OCT compound, frozen, and sectioned (10 μm). Sections were hybridized with antisense digoxygenin-labeled cRNAs corresponding to full-length hGH mRNA according to the manufacturer's instructions (Boehringer Mannheim).

Skin Grafting. Grafting was performed as described (22). Briefly, tailskins were separated from two month old line 22 transgenic mice, and reserved in culture medium while severe combined immunodeficient (SCID) mice were prepared as recipients of the grafts. SCID mice were anesthetized with Nembutol and ≈1.5-cm^2 strips of their backskins were peeled back from the muscle fascia. These denuded areas were then grafted with equivalent sections of line 22 tailskin. The healing process took ≈2 weeks, after which time serum aliquots were periodically drawn from the eyes of these animals. Sera were tested for the presence of hGH as outlined above.

RESULTS

The K14 Promoter Is Largely Restricted to Skin Keratinocytes, Making it Feasible to Use K14 Promoter-Driven Transgenes in Mouse Models for Keratinocyte-Mediated Gene Therapy. A prerequisite for using transgenic mice in evaluating the potential of skin keratinocytes for gene therapy is the availability of a well-characterized promoter that has an expression pattern restricted largely to skin keratinocytes. Previously, we had shown that 2300 bp of the human K14 promoter could be used to express an epitope-tagged K14 protein in the skin, tongue, and esophagus of newborn transgenic mice (16). To monitor the tissue specificity of the K14 promoter independent of K14 coding and noncoding sequences, we engineered a mammalian K14 expression vector and used a short-lived β-gal transgene as a reporter (see Fig. 1). The pattern of expression of β-gal, assayed by X-Gal conversion (Fig. 2), was compared with that of endogenous K14, assayed by immunohistochemistry (not shown). These data are compiled in Table 1.

Table 1. Distribution of K14 in adult mouse tissues

Tissue	K14 (endogenous)	K14 promoter (transgene expression)	RT-PCR
Complex epithelia			
Epidermis	++++	++++	++++
Palmoplantar skin	++++	++++	
Hair follicle (ORS)	++++	++++	
Cornea	+++	+++	
Oral epithelium	+++	+++	
Tongue	++	++	++
Esophagus	+	−	
Forestomach	+	−	−
Salivary glands	+	+/−	
Mammary epithelium	+	+/−	+
Cervix/vagina	+	+/−	
Thymus	+	+/−	+
Trachea	+	−	
Bladder	−	−	
Pancreas	−	−	
Simple epithelia			
Kidney	−	−	
Thyroid	−	−	
Seminal vesicle	−	−	
Ovary	−	−	
Small intestine	−	−	
Gallbladder	−	−	
Oviduct	−	−	
Colon	−	−	
Uterus	−	−	
Nonepithelial			
Liver	−	−	
Adrenal glands	−	−	
Testis	−	−	
Habenular of brain	−	*	
Rest of brain	−	−	−

Tissues from adult K14-βgal or K14 expressing transgenic mice were frozen in OCT compound, sectioned, and either assayed for β-gal activity or subjected to immunohistochemistry using an anti-K14 antibody or an anti-P antibody. In addition, RNAs were isolated from some tissues and detection of transgene mRNA was determined by RT-PCR. *, A very faint signal, barely detectable, was seen after overnight incubation. +/−, Very patchy staining where only a small percentage of the epithelial cells were positive for transgene expression, and expression was markedly less than seen in the epithelial cells of the skin.

In skin and oral epithelia where K14 expression is known to be high (13, 23, 24), 2100 bp of the K14 promoter faithfully directed abundant expression of β-gal (Table 1; see examples of data in Fig. 2). As expected, β-gal was expressed in the basal layer of epidermis (Fig. 2*A*) and in the outer root sheath of the hair follicle (not shown), where endogenous K14 is known to be expressed (23). It was also expressed in tongue (Fig. 2*B*) and in buccal epithelium (not shown). These findings were consistent with previous data obtained on K14 promoter-expressing transgenic mice (16).

Unexpectedly, β-gal activity became increasingly patchy, aberrant, and/or reduced in more internal stratified tissues. In

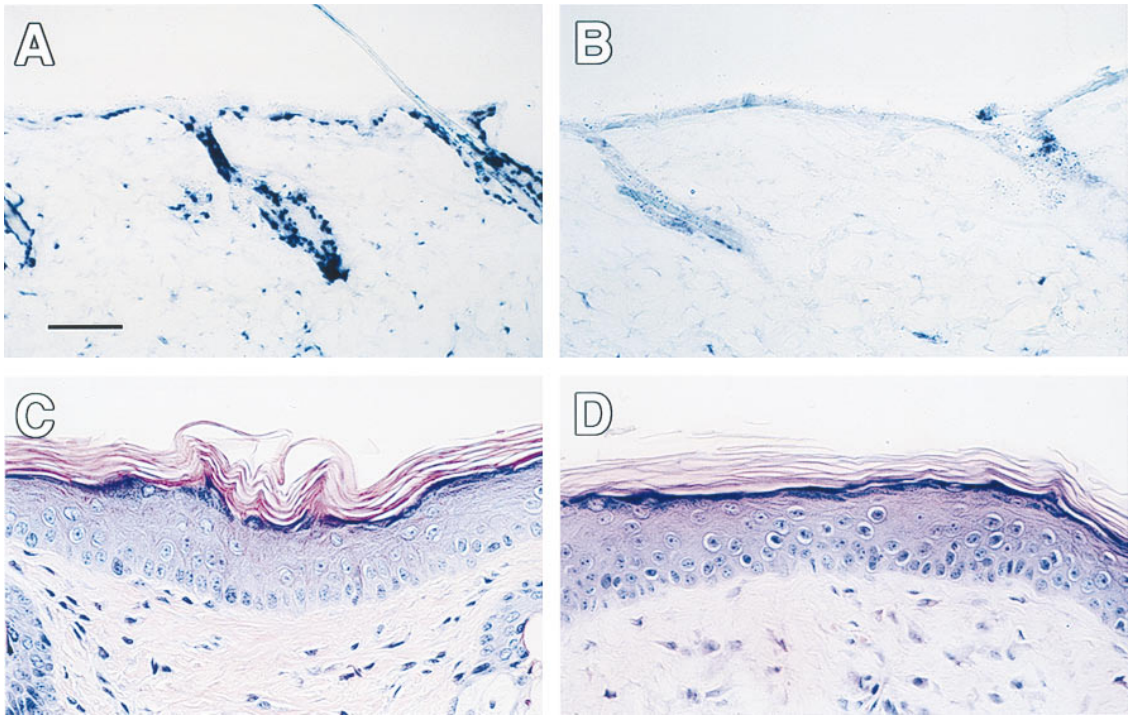

FIG. 3. Ectopic expression of the hGH transgene in skin does not alter epidermal morphology. (*A* and *B*) Backskin sections from an adult line 22 K14–hGH expressing mouse (*A*) and a control littermate (*B*) were subjected to *in situ* hybridization with an antisense digoxygenin-labeled hGH cRNA. (*C* and *D*) Tailskin sections from an adult transgenic (*C*) and control (*D*) mouse were stained with hematoxylin/eosin. Note that the epidermis of backskin, with abundant hair follicles, is thinner than that of tailskin, with few follicles. (Bar = 90 μm in *A* and *B*, and 45 μm in *C* and *D*.)

esophagus, trachea, and forestomach, β-gal activity was not detected at all (Fig. 2 *C* and *D*). In other internal tissues, transgene expression was only detected in a small percentage of the epithelial cells within the tissue (Fig. 2 *E* and *F*). These findings were consistently observed in independently derived lines of transgenic mice and in animals examined at various ages. Thus, transcriptional activity of the 2100-bp transgene promoter seemed to be largely restricted to external epithelia, despite expression of endogenous K5 and K14 in internal stratified squamous epithelia (13, 24, 25). The activity of the K14 promoter segment appeared to be more restricted than that previously reported for 6000 bp of K5 promoter (26, 27). These results enabled us to consider a transgenic approach to further explore the potential of the 2100-bp human K14 promoter for future keratinocyte-based gene therapy.

The Use of the K14 Promoter to Drive Selective and Sustained Expression of Human Growth Hormone in the Skin of Transgenic Mice. To test the ability of skin keratinocytes to deliver secreted products such as hGH to the bloodstream, we engineered mice harboring the K14 promoter–hGH transgene illustrated in Fig. 1. Ten founder mice tested positive for the transgene, as judged by PCR analysis of their ear DNAs. All founder animals and their F_1 offspring behaved similarly, except for the degree to which phenotypic abnormalities (discussed later) were observed. Of these, the 22 and 39 mice lines displayed the strongest phenotypes, and they were used for subsequent studies. Only male mice appeared fertile; attempts to breed female animals failed, consistent with previous reports on other types of transgenic mice engineered to overexpress growth hormone (28–30).

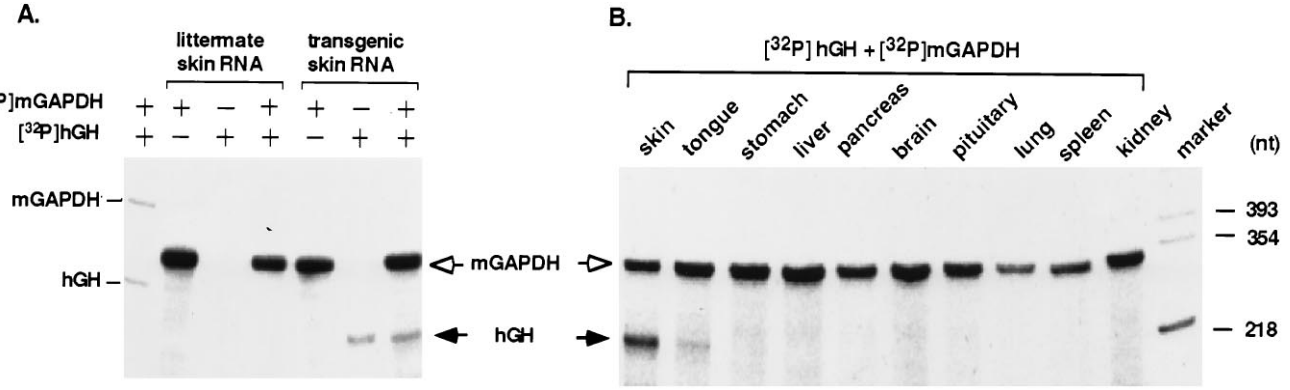

FIG. 4. RNase protection assays to quantitate hGH RNA levels in transgenic mouse tissues. RNase protection assays were carried out as described. (*A*) Assays on adult skin. First lane shows migration of aliquot of antisense RNAs before RNase digestion. Other lanes show migration of radiolabeled antisense probes after incubation with skin RNAs and RNase treatment. Note the reduction in size for both mGAPDH (open arrowhead at right) and hGH (filled arrowhead at right) RNAs, reflective of digestion of the random (i.e., nonhybridizing) sequences in each of the two probes. This serves as an internal control for the RNase. (*B*) Assays on tissues. Lanes show protected radiolabeled cRNAs after hybridization with 9-month-old transgenic tissue RNAs and subsequent RNase treatment. Last lane shows migration of *Hin*fI-digested pSP64 plasmid DNAs, end-labeled and used as molecular mass markers. (Note, the specific activity of the mGAPDH probe was higher in *A* versus *B*.)

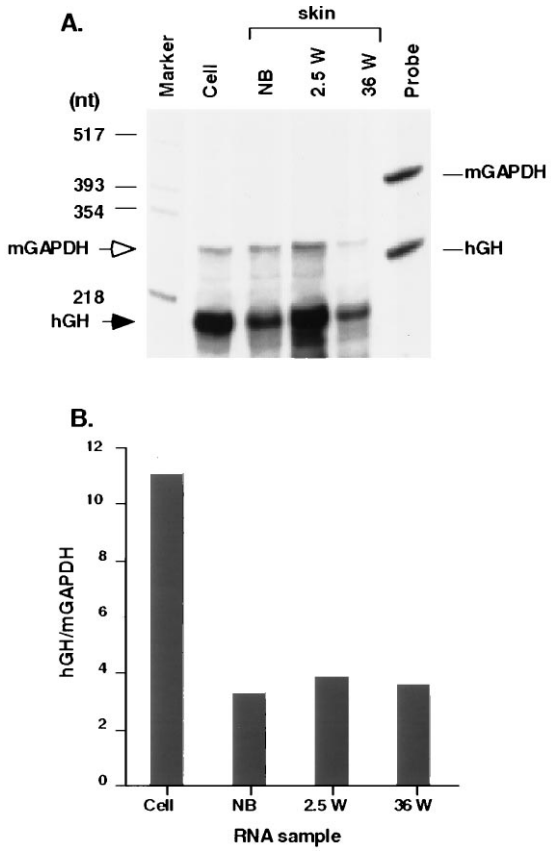

FIG. 5. RNase protection assays to quantitate hGH RNA levels in transgenic mouse skin at different ages. (*A*) RNAs were isolated from transgenic skin at birth (newborn, NB), and at 2.5 and 36 weeks postnatally. RNAs were also isolated from cultured transgenic mouse keratinocytes (Cell). RNase protection assays were carried out as described in the legend to Fig. 3, except that the specific activity of the radiolabeled mGAPDH antisense probe added to each sample was less than that used previously. Following hybridization and RNase treatment, the protected RNA fragments were resolved by electrophoresis through 6% acrylamide gels containing 8 M urea. (*B*) Phosphoimage analysis of blot in *A*. The amount of hybridizing radioactivity in each band was determined to obtain the ratio of protected hGH RNA relative to internal mGAPDH RNA in each sample. Relative to the level of mGAPDH RNAs in skin, the level of hGH transgene RNA did not vary significantly over time.

In situ hybridization showed that the hGH transgene was expressed in the basal layer of the epidermis and in the outer root sheath of the hair follicle (Fig. 3*A*; control in *B*). This was confirmed by immunohistochemistry with an anti-hGH antibody (not shown). Even our highest expressing lines of transgenic mice displayed no major perturbations in epidermal morphology (Fig. 3*C*; control in *D*). This was true in both younger (shown) and older (not shown) skin. The only minor change that we detected in the skin was a slight shortening of the hair coat, suggesting a very mild alteration in the hair cycle.

Table 2. hGH levels in mouse sera and keratinocyte culture media

Serum hGH, ng/ml		Culture media hGH,
2.5 weeks	36 weeks	ng/10^6 cells/day
11 ± 2	49 ± 6	251 ± 16

hGH levels were determined using a RIA as described. At times indicated after birth, blood from K14–hGH transgenic mice was removed from the eye vein with a hypodermic syringe. Diploid cultured keratinocytes were prepared from newborn transgenic mouse skin as described (19). Each assay was performed in duplicate or triplicate on material from 3–6 animals/cultures.

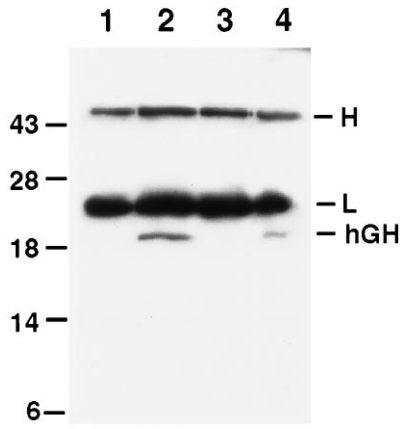

FIG. 6. Immunoblot analysis of anti-hGH immunoprecipitates from sera of hGH transgenic mice and from the spent medium of keratinocytes cultured from newborn animals. Sera were taken from the bloodstream of 9-month-old transgenic and control mice. Keratinocytes from newborn control and transgenic mice were cultured in duplicate as described; at 80% confluence, fresh medium added, and the medium was sampled after an additional 24 hr of culture. Immunoprecipitations to detect the hGH transgene product in the skin or in the culture medium were carried out using mAb-coated plastic beads for hGH (Hybritech) under the same conditions as RIAs (see *Materials and Methods*), but without radiolabeled tracer. Aliquots of the immunoprecipitates were then resolved by SDS/PAGE and subjected to immunoblot assays, performed with a mAb against hGH (Sigma). Immunoprecipitates were from control serum (lane 1), transgenic serum (lane 2), control medium (lane 3), and transgenic medium (lane 4). Migration of molecular mass standards are indicated at left in kilodaltons; migration of heavy (H) and light (L) chains of γ-immunoglobulin is given at right.

Thus, overall, the K14–hGH transgene did not appear to affect significantly the tissues in which it was expressed.

To further quantitate transgene expression levels, we used RNase protection assays. Skin RNAs from 36-week-old transgenic and control mice were hybridized with a radiolabeled hGH probe, followed by RNase treatment. As shown in Fig. 4*A*, a band of 207 nucleotides was protected in the transgenic but not the control skin RNA sample. The level of transgene RNA in skin of adult mice was much higher than that in internal stratified tissues, including tongue (Fig. 4*B*), confirming the qualitative data obtained from our β-gal transgene studies. As expected, nonepithelial tissues were negative for transgene expression.

To determine if the K14 promoter remained active in older mice, we performed RNase protection assays on skin RNAs isolated from line 22 transgenic mice at birth and at 2.5 and 36 weeks of age. As shown in Fig. 5, the level of hGH mRNA

FIG. 7. K14–hGH transgenic mice are larger than control animals. Shown are a 2-month-old K14–hGH transgenic mouse (*Left*) and its control littermate (*Right*) from line 22. Note the increase in body size of the hGH-expressing mouse.

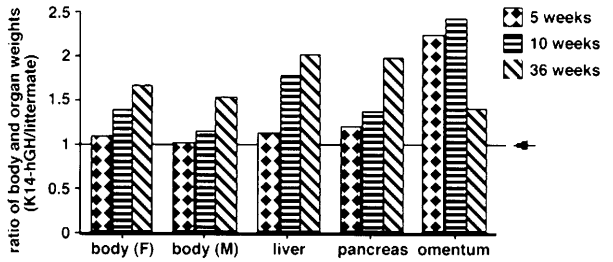

FIG. 8. Weights of K14–hGH transgenic and control animals and organs. Male K14–hGH transgenic and control littermates were killed and/or weighed at the ages indicated. Organs were removed and weighed from 3–5 animals in each group, and an average weight was calculated. Shown is a plot of the ratio of transgenic versus control body and organ weights for each age group. Arrow denotes control level (ratio = 1). Because transgenic line 22 and 39 did not vary significantly in their organ weights, the values shown represent a combination of the measurements on the two lines. Shown for comparison are the data from MtI–hGH transgenic mice reported previously by Palmiter *et al.* (32, 40).

remained constant over time, relative to an internal control amount of GAPDH mRNA in skin.

High Levels of Processed hGH in the Circulation and Epidermal Keratinocytes of K14–hGH Transgenic Mice. To assess whether the hGH produced by K14–hGH transgenic skin is appropriately processed, secreted and transported to the bloodstream, we used RIAs to measure serum levels of hGH (Table 2). As expected, hGH was not detected in serum from control littermates, in agreement with the species specificity of the assay. In striking contrast, the circulatory levels of hGH ranged from ≈25 to 50 ng/ml in adult mice from the two different hGH lines examined. This level was ≈1000-fold higher than that of endogenous mouse GH (31). It was comparable to that obtained in transgenic mice expressing metallothionine I (MtI) promoter-driven hGH (32) and higher than that seen in mice expressing hGH driven by the mouse mammary tumor virus long terminal repeat enhancer (33). The serum levels of hGH were consistently higher in adult animals than in juvenile (2.5 week) mice, perhaps reflective of the increase in skin surface area relative to blood circulation in adult animals. Importantly, these data demonstrate that the K14 promoter is capable of delivering high levels of hGH to the bloodstream throughout the life of the animal.

To verify that the hGH detected in the circulation had been processed correctly and represented the mature form, we immunoprecipitated serum proteins with anti-hGH antibody, resolved the precipitates by electrophoresis through SDS polyacrylamide gels, and conducted immunoblot analysis with an anti-hGH antibody. As shown in Fig. 6, a band of the expected size of 22 kDa was present in the serum of transgenic, but not control animals.

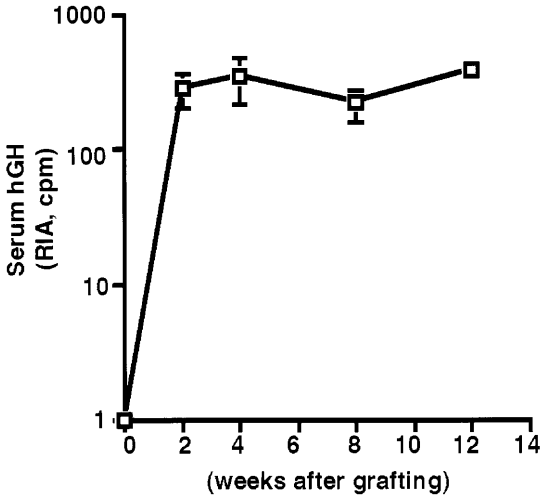

FIG. 9. Detection of hGH in the bloodstream of SCID mice grafted with a patch of K14–hGH tailskin. The tailskins of 2-month-old K14–hGH transgenic mice were removed and grafted in 1–2 cm^2 segments onto the backs of six recipient immunodeficient SCID mice. Animals were allowed to recover for 1–2 weeks, after which time blood was analyzed by RIA for hGH. To collect sufficient blood at 1-week intervals, samples from two animals were often combined for the assay, yielding three measurements from each week. The variation in levels are indicated by the vertical bars. To date, animals have been monitored for 14 weeks after the graft. After an initial recovery period after grafting, the concentrations of serum hGH were consistently within the range of 0.1–0.4 ng/ml.

To examine the production of hGH by transgenic epidermal cells, we cultured newborn mouse skin under conditions that favored keratinocyte growth (19). Before confluence, keratinocytes were tested for their ability to produce, process, and secrete hGH into the culture medium. As shown in Table 2, cultured keratinocytes produced very high levels of secreted hGH (>200 ng hGH/10^6 cells per day). This hGH was processed correctly, as judged by immunoblot analysis of anti-hGH immunoprecipitated proteins from the culture medium (Fig. 6).

Assuming that there are ≈10^8 K14-expressing keratinocytes in the skin of an adult mouse, we estimate that >20 μg of hGH per day could be produced by the epidermal keratinocytes of these transgenic mice. Estimating ≈5 ml of total serum in an adult animal, at least 1% of the hGH produced by keratinocytes can survive in the circulation. Given the known instability of hGH (34, 35), this level is appreciable. It remains unknown whether the remainder of the hGH produced is degraded in the bloodstream or never makes its way across the basement membrane and into the circulation.

Physiological and Metabolic Changes in Mice Expressing the K14–hGH Transgene. Of founders harboring the K14–hGH

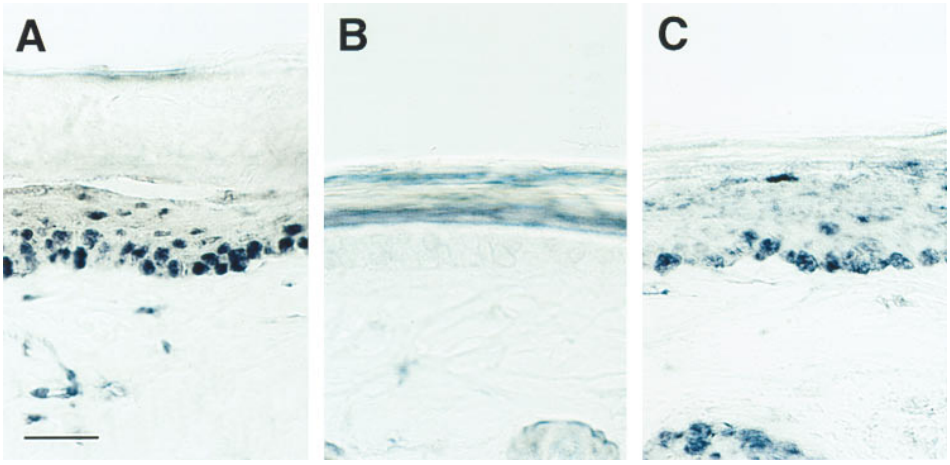

FIG. 10. *In situ* hybridization revealing hGH mRNA in the epidermis of K14–hGH skin grafts. A skin biopsy from one of the tailskin grafts of a SCID mouse recipient was removed and processed for *in situ* hybridization with a digoxygenin-labeled antisense hGH cRNA probe. (*A*) Biopsy from tailskin graft. (*B*) Control tailskin. (*C*) Tailskin from a K14–hGH transgenic mouse. Note that the dead, s. corneum layer occasionally traps some labeling (see example in *B*), but this is an artifact and is not consistently observed. Note the complete absence of labeling in the living epidermal layers of control skin (*B*). Note that the cells in the dermis of *A* and *C* that show labeling are from segments of hair follicles. (Bar = 30 μm.)

transgene, mice appeared normal at birth, but with age, they all began to show morphological differences distinguishing them from normal mice (Fig. 7). The majority of the physiological and metabolic responses were ones well known as direct or indirect responses of growth hormone (32, 37–40). Within 10 weeks of age, both male and female transgenic mice had gained weight relative to their age and sex matched littermates (Fig. 8). By 36 weeks of age, these mice were an average of 1.6 times the size of control mice, similar to mice expressing the MtI promoter-driven rat growth hormone (MtI–rGH; ref. 41). A good correlation existed between the level of hGH expressed by a line and its relative increase in body mass by 36 weeks of age. The weight gain trait was inherited by the F_1 offspring, and it was not dependent upon transgene insertion site.

The similarities between our K14–hGH transgenic animals and MtI–rGH mice (6, 41) extended to organ size and tissue weight. Similar to that seen in the MtI–rGH mice (41), the liver, pancreas, and omentum of 5- to 36-week-old K14–hGH mice were significantly larger than normal (Fig. 8). In contrast, increases in brain heart, lungs, spleen, and kidneys were more modest (data not shown).

The effects of growth hormone extended to insulin production, a well-known secondary response (42–44). At 5 weeks of age, circulating insulin levels were already twice that of wild-type control animals, and by 36 weeks of age, these levels (up to 350 microunits/ml) had risen to nearly 10-fold the control values. These values were similar to those seen in mice expressing a murine sarcoma virus promoter–hGH transgene (33).

In contrast to the high levels of insulin, serum glucose levels were not appreciably different between control and K14–hGH transgenic animals (147 ± 27 mg/dl, control versus 156 ± 13 mg/dl, transgenic; 36 weeks). However, upon intraperitoneal administration of 2 μl of a 50% glucose solution per gram body weight, animals that had been fasted for 4 hr showed a markedly altered glucose tolerance response (150 ± 15 mg/dl, control versus 325 ± 15 mg/dl, transgenic; 36 weeks). The inability to properly regulate glucose is likely to be a secondary response, reflective of the high insulin levels of these animals.

K14–hGH Transgenic Skin Grafts Give Rise to Circulating hGH in Nontransgenic Recipient Mice. Of key importance in assessing the potential relevance of our study to gene therapy is to determine whether a skin graft from a K14–hGH expressing mouse can deliver systemic hGH to a recipient nontransgenic mouse. To examine this possibility, we removed the tailskins from line 22 transgenic mice and grafted them onto the backs of immunodeficient (SCID) animals. Within a few weeks after grafting, circulating hGH could be detected (Fig. 9). Depending on the animal and size of the graft, levels ranged from 0.15 to 0.40 ng/ml, or ≈0.15 ng/ml for each cm^2 of skin graft. While these levels were only ≈1/10 physiological levels, the hGH levels remained stable after the initial period while the animals were recovering from the surgical operations. *In situ* hybridization confirmed that growth hormone gene expression persisted in the skin grafts (Fig. 10). These data demonstrated that the human K14 promoter remained active in adult skin even after grafting.

DISCUSSION

Given their extraordinary proliferative capacity in culture (1–3), and the well-documented value of cultured keratinocytes in burn grafting operations (4), genetically manipulated skin keratinocytes have long been proposed as a potential vehicle to correct for a human deficiency in a circulating protein or factor (5, 12, 45–47). In the first systemic delivery experiments attempted, Morgan *et al.* (5) grafted cultured retroviral promoter-driven hGH-transfected keratinocytes to the skin of a nude mouse. While these keratinocytes produced ≈70 ng per 10^6 cells per day in culture, no hGH was detected in the serum of mice harboring skin grafts from these cultures. Subsequent attempts to improve hGH expression in grafts involved the use of the Herpes thymidine kinase, MtI and Epstein–Barr virus promoters, and while an initial serum level of a few ng/ml hGH was detected, hGH was not detected several weeks after the graft was applied (11, 12). Similar studies were obtained by Gerrard *et al.* (48), who used the Moloney murine leukemia virus to express human factor IX; at 1 day after grafting, factor IX could be detected at 3 ng/ml in the serum, but 6 days later, it was no longer detectable.

Based on our present studies, we have now ruled out a major caveat for the failure to detect appreciable levels of circulating hGH or human factor IX in grafted mice; namely, that keratinocytes are unable to efficiently transfer these factors to the circulation. Rather, as was observed by Fenjves *et al.* (6, 47) for apolipoprotein E, by Cheng *et al.* (49) for tumor necrosis factor α (TNF-α), and by Mathor *et al.* (50) for interleukin 6, hGH can be secreted by a stratified epithelium, and traverse the basement membrane to enter the blood vessels in the underlying mesenchyme.

The experiments conducted by Teumer *et al.* (11), Gerrard *et al.* (48) and Jensen *et al.* (12) all suggest strongly that keratinocytes expressing transgenes driven by foreign promoters are unlikely to produce sustained transgene expression following grafting *in vivo*. Of all keratinocyte grafting experiments conducted to date, only Fenjves *et al.* (47) were able to detect systemic delivery of a foreign transgene product 4 weeks after grafting to nude mice. Because the ability of the graft to produce circulating apolipoprotein E was not studied over time, it remains to be shown whether the SV40 promoter/enhancer can maintain long-term, high expression of a transgene in grafted keratinocytes.

Our first indication that the K14 promoter might be useful for keratinocyte-derived delivery of factors to the bloodstream came from our detection of TNF-α in the serum of K14–TNF-α transgenic mice (49). These animals died within several weeks after birth, making it impossible to assess the long-term potential for keratinocyte-derived K14–TNF-α to sustain high serum levels of the transgene product. The bovine K10 promoter, expressed in terminally differentiating keratinocytes, has also been used to produce and secrete high levels of circulatory factors in transgenic mice (51, 52).

To use transgenic animals as a system to explore the potential efficacy of keratinocyte-mediated gene therapy, it is essential to have a strong promoter that is largely restricted to the skin in its activity and that maintains its activity in adult skin. To be feasible for genetic manipulation of cultured human keratinocytes, the promoter must also be strongly active in cultured cells. Of the epidermal promoters studied to date, the 2100-bp K14 promoter segment is the only one that fully meets these criteria.

In the present study, all 10 lines of K14–hGH mice expressed sufficiently high levels of circulatory hGH to elicit marked physiological and metabolic changes over time, including primary and secondary responses characteristic of GH. The circulatory levels of keratinocyte-derived, secreted hGH remained high in adults, and grafting studies further underscored the suitability of the 2100-bp human K14 promoter for driving long-term expression of a circulatory factor by skin grafts. The accessibility of keratinocytes, their marked proliferative capacity in culture, and their ability to be maintained as localized patches of a selected size on the body surface provide them with certain potential advantages over other cell types that have been tested as possible vehicles for hGH delivery (53–55).

This said, there are still a number of key issues that remain to be addressed before keratinocytes can be considered as a plausible vehicle for gene therapy. One limitation to our studies is that mice receiving a patch of K14–hGH that covered ≈1–5% of the body surface only produced circulatory levels of hGH that were within an order of magnitude of that expected for a normal growing child. In addition, even though tailskin was used to minimize the contribution of hGH gene expression from the outer root sheath of hair follicles, our studies were

nevertheless conducted with whole skin and not pure epidermis. To be feasible for gene therapy of cultured keratinocyte grafts, the activity of the promoter may need to be increased, something which should be possible as we learn more about the regulatory elements controlling K14 gene expression. Another key issue to address in the future will be one of gene transfection efficiency. Using other cell types, the most successful gene therapy approaches have been those employing retroviral or adenoviral delivery systems (56, 57). We have not yet explored various delivery systems with keratinocytes, and for our studies to be relevant to gene therapy, we will need to be able to efficiently transfect our keratin promoter-driven transgene into cultured keratinocytes for subsequent grafting.

In summary, our studies have shown that the K14 promoter maintains strong activity in skin keratinocytes *in vivo* and *in vitro*, and it is largely, albeit not solely, independent of its chromosomal position. The activity is sufficient to enable a skin patch to generate within an order of magnitude the circulatory levels of GH that are normally expected of the pituitary gland. These data indicate that if effective methods are now devised to stably transfect human skin keratinocytes in culture, or perhaps *in situ*, keratinocyte-mediated gene therapy could become a powerful tool in human medicine, for correcting either genetic skin disorders or systemic deficiencies.

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